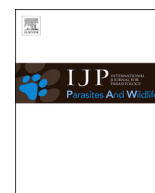


Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Sarcocystosis in *Cervus elaphus*: Comparison of diagnostic methods



Mónica Luzón^{*}, Julia Domínguez-González, Ana María Soto-Carrión, José María Alunda, Concepción de la Fuente

Department of Animal Health, Veterinary Faculty, Complutense University of Madrid, Avenida de Puerta de Hierro s/n, 28040 Madrid, Spain

ARTICLE INFO

Article history:

Received 14 July 2015

Received in revised form

30 October 2015

Accepted 5 November 2015

Keywords:

Sarcocystis

Cervus elaphus

Diagnostic: compression/histology

Heart/diaphragm

ABSTRACT

Red deer (*Cervus elaphus*) from a National Wildlife Reserve near Toledo in central Spain were surveyed for *Sarcocystis* infection. A total of 61 deer were examined. Tissue compression and histology were used to examine samples from diaphragm and heart from each animal included in the study, and results from the two techniques and the two tissues were compared to determine the tissue and technique that provide the most accurate measure of prevalence and intensity. Prevalence and intensity were then compared between calves, yearlings and adults. *Sarcocystis* was detected in 59 (97%) of the 61 deer. Comparison between tissues showed that (a) prevalence based on histology was similar for heart and diaphragm, (b) prevalence based on compression was significantly higher for heart than for diaphragm and (c) intensity was significantly higher for heart than for diaphragm, regardless of the technique used. Comparison between techniques showed that (a) both techniques rendered similar prevalences and intensities of *Sarcocystis* infection with heart samples and (b) both techniques were not comparable with diaphragm samples (compression rendered lower prevalence but higher intensity than histology). Together these data suggest that heart is the preferable tissue for estimating prevalence and intensity, regardless of the technique used. A preliminary species identification of isolated cysts from three animals showed two morph types, corresponding to *Sarcocystis cervicanis* (syn. *S. cf. grueneri*; *S. wapiti*) in the heart and diaphragm of three animals and *S. hjorti*, only in the diaphragm of two animals. Given the different location of those morph types, both heart and diaphragm should be sampled and preferably assessed using histology to most reliably detect infection. Based on histology of heart, prevalence and intensity of *Sarcocystis* were significantly lower in calves than in yearlings or adults.

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1. Introduction

Wild ungulates, including red deer (*Cervus elaphus*), are an essential component of the environment in Europe and North America. Their populations have an important role in the preservation of biodiversity and have a substantial economic impact in many areas (e.g. recreational purposes, hunting activities). Different surveys have shown that red deer are affected by a variety of parasitic agents (Hernández-Rodríguez et al., 1981). In spite of limited evidence it is generally considered that these infections, individually and in combination, have a negative effect on the performance of infected deer.

Sarcocystosis is one of the most widespread tissue parasitoses of domestic and wild herbivores and several studies indicate that the

prevalence of sarcocystosis in red deer is very high (Partenheimer-Hannemann, 1991; Malakauskas and Griekienienė, 2002; Spickschen and Pohlmeier, 2002; López et al., 2003). Despite abundant information about *Sarcocystis* infection in red deer in Europe and North America, data for prevalence and intensity of infection are rarely comparable, mainly due to the different methodologies applied (compression, histology, tryptic digestion) (Entzeroth et al., 1983; Kutkienė, 2003), the muscle tissue selected for the analysis (e.g. heart, diaphragm, oesophagus), the age of the sampled animals (Kutkienė, 2003) and the tropism attributable to some *Sarcocystis* spp. (Kutkienė, 2003; Dahlgren and Gjerde, 2010).

In the present work we present the results of a survey on sarcocystosis in red deer from central Spain with the aim of: 1) comparing the accuracy of detection using two muscles (heart, diaphragm) and two techniques (histology, compression); 2) evaluating the degree of correlation and agreement between muscles and techniques, and 3) determining the influence of deer age on the prevalence and/or intensity of *Sarcocystis* infection. In addition, a

^{*} Corresponding author.

E-mail address: mluzon@ucm.es (M. Luzón).

preliminary identification of the *Sarcocystis* spp. was made based on morphological criteria, i.e. thickness of cyst wall and absence/presence/appearance of surface protrusions, according to the cyst descriptions of Hernández-Rodríguez et al. (1981) for *Sarcocystis cervicanis* and Dahlgren and Gjerde (2010) for the other *Sarcocystis* spp.

2. Materials and methods

2.1. Sampling and analytical techniques

Samples were collected in the Quintos de Mora park (Toledo, Central Spain, 39° 25'N, 4° 04'W) from National Wildlife Reserves. The presence of cysts of *Sarcocystis* was investigated *post mortem* in 61 red deer (9 calves, 15 yearlings, 37 adults) shot in selective hunting from June 2005 to October 2006. Muscle samples of heart (inter-ventricular septum) and diaphragm (diaphragmatic pillars) were collected. Whenever possible, duplicate muscle samples were taken: one sample was kept at -20°C and the other was placed in buffered formalin (10% formalin in phosphate buffered saline) for compression and histology techniques, respectively. Compression analysis was based on the classical trichinoscopic technique for detection of *Trichinella* infection in food animals according to the OIE Manual of Standards for Diagnostic Tests and Vaccines (OIE, 2004). Accordingly, 28 thawed, oat-grain-size samples (ca. $2\text{ mm} \times 10\text{ mm}$) of a selected muscle were compressed between two glass plates of a trichinoscope (Nahita, Auxilab, Beriain, Spain) until they became translucent and were examined for *Sarcocystis* cysts using a conventional light microscope (Eclipse E–100, Nikon, Tokyo, Japan) at $100\times$ magnification. The intensity was expressed as cysts/28 samples (estimated area equivalent to 5.6 cm^2). Histological analysis was done on $3\text{--}5\text{ }\mu\text{m}$ sections stained with haematoxylin-eosin. For each sample, an area of 1 cm^2 was analysed at $100\text{--}400\times$ magnification, and the intensity of infection determined (cysts/ cm^2).

Of the 61 animals, 59 were analysed by histology of heart; 53 by compression of heart; 49 by histology of diaphragm, and 47 by compression of diaphragm. Only animals with paired samples (both techniques and both muscles) were considered for comparisons. Intensity of infection in each animal was classified as low (<10 cysts/ cm^2 by histology or in the 28 samples examined by compression), moderate (10–40 cysts) and high (>40 cysts) following Kutkienė (2003). Prevalence, mean intensity and median intensity were calculated for both analytical techniques in the two muscles studied. These measures are considered suitable descriptors to quantify parasites in a sample of hosts (Rózsa et al., 2000).

To ascertain the presence of multispecific infections, samples from three animals with high *Sarcocystis* intensity were selected and a total of 59 *Sarcocystis* cysts (15 from heart and 44 from diaphragm) were examined under stereoscope (Stereoscopic Zoom Microscope, Nikon) and isolated from muscle using dissecting forceps and a fine needle, then examined under a Nomarski microscope (Nomarski Eclipse 80 i, Nikon) ($100\times\text{--}400\times$) and morphologically classified following the cyst descriptions of Hernández-Rodríguez et al. (1981) for *S. cervicanis* and Dahlgren and Gjerde (2010) for the other *Sarcocystis* species.

2.2. Statistical analysis

Comparisons of prevalence in different muscles and with the two techniques and between age groups were performed with the Upton's χ^2 test ($N - 1\text{ } \chi^2$ test). Agreement in the detection of cysts between techniques and between muscles was analysed using Cohen's kappa coefficient. Since parasites tend not to have a normal

distribution within host populations, comparisons of intensities of infection (techniques; muscle regions; age groups) were carried out with the Wilcoxon test and correlations between techniques and between analysed tissues were determined using the Spearman's rank correlation coefficient (r_s). All statistical procedures were performed with the WinPEPI® 7.5 Software for Windows.

3. Results

No significant differences in prevalence of *Sarcocystis* infection were observed between muscles or techniques (values between 76%–81%) except with compression of diaphragm, which yielded significantly lower values than those found with compression of heart (50% vs 81%, $P < 0.01$) and with histology of diaphragm (53% vs 77%, $P < 0.05$) (Table 1). Considering the overall prevalence irrespective of the technique or muscle selected, *Sarcocystis* infection was detected in 97% (59 of 61) of the sampled animals.

Low intensities were predominant in all samples (87% in histology of diaphragm; 65% and 81% in compression of heart and diaphragm, respectively) except when the heart was studied by histology (51%). Median intensities of infection were significantly higher in heart using histology ($P < 0.01$) or compression ($P < 0.05$) (Table 2). The highest intensity was detected in an animal with 109 cysts/ cm^2 (heart) and 99 cysts/ cm^2 (diaphragm) (data not shown). Comparison of median intensities between both techniques (analysing 1 cm^2 of sample by histology vs 5.6 cm^2 by compression) showed apparently similar results with heart samples, whereas compression of diaphragm samples yielded a higher median intensity ($P < 0.01$) (Table 3).

Intensities of *Sarcocystis* infection were moderately correlated between techniques in both the analysis of heart ($r_s = 0.58$; $P < 0.01$) and diaphragm ($r_s = 0.59$; $P < 0.01$), whereas correlation between muscles was weak using any of the techniques (histology: $r_s = 0.24$; $P < 0.05$; compression: $r_s = 0.31$; $P < 0.05$) (Table 4). Similarly, the agreement in the detection of cysts (positive vs negative) between techniques was moderate in heart (kappa = 0.42; $P < 0.01$) and diaphragm (kappa = 0.40; $P < 0.01$), and no agreement in the detection of cysts was observed between muscles using either technique (Table 5).

Comparison between ages using the relatively sensitive combination histology – heart showed that the number of calves infected was significantly lower than those of yearlings ($P < 0.05$) and, particularly adults ($P < 0.01$). Similarly, median intensity of infection was also lower in calves than in older animals ($P < 0.05$) (Table 6).

In the preliminary analysis of cyst morphology two morph types of *Sarcocystis* were found: one with a thin wall without apparent protrusions, identified as *S. cervicanis* (syn. *S.cf. grueneri*; *S. wapiti*), and other showing a thin wall with densely arranged, hair-like, surface protrusions which were only apparent after cyst isolation, identified as *S. hjorti*. All cysts detected in heart and approximately 70% of those in diaphragm were identified as *S. cervicanis*; the remaining 30% of cysts detected in diaphragm were identified as *S. hjorti*. Mixed infections were noted in two of the animals whereas in one animal only *S. cervicanis* was identified.

4. Discussion

Overall results indicate that *Sarcocystis* infection of red deer was highly prevalent (97%) in the area. Results obtained after analysing paired red deer samples (diaphragm, heart) with two techniques (compression, histology) showed wide variations in prevalence and intensity of *Sarcocystis* infection. Prevalence of *Sarcocystis* in heart (76%–81%) was similar to or higher than that in diaphragm, ranging from 50% to 80% depending on the technique employed;

Table 1

Comparison between prevalence of infection by *Sarcocystis* sp. detected in two muscle tissues (heart vs diaphragm) and analysed by two different techniques (histology vs compression).

	Prevalence n/N % (95% C.I.)		Statistical significance (Upton's Chi square test)
	Heart	Diaphragm	
Histology (N = 49)	37/49 76% (62–86)	39/49 80% (67–89)	–
Compression (N = 42)	34/42 81% (67–91)	21/42 50% (35–65)	
	Histology	Compression	
Heart (N = 51)	39/51 76% (63–87)	41/51 80% (68–90)	–
Diaphragm (N = 47)	36/47 77% (63–87)	25/47 53% (39–67)	
			P < 0.01
			P < 0.05

C.I.: confidence interval.

–: No statistical significance.

Table 2

Comparison between the intensities^a of infection by *Sarcocystis* sp. detected in two muscle tissues: heart vs diaphragm, according to the technique.

	Intensity % low; % moderate; % high Mean ± SD Median		Statistical significance (Wilcoxon test)
	Heart	Diaphragm	
Histology (n = 49)	51%; 41%; 8% 17.5 ± 22.3 9	87%; 10%; 3% 7.1 ± 16.6 2	P < 0.01
Compression (n = 42)	65%; 29%; 6% 12.8 ± 14.9 6	81%; 19%; 0% 5.5 ± 4.0 4	
			P < 0.05

SD: standard deviation.

^a Intensities are classified as low (<10 cysts), moderate (10–40 cysts) or high (>40 cysts) and expressed as percentages.

Table 3

Comparison between intensities of infection by *Sarcocystis* sp. detected by histology vs compression, according to the muscle analysed.

	Intensity (Mean ± SD) Median		Statistical significance (Wilcoxon test)
	Histology	Compression	
Heart (n = 51)	(15.4 ± 21.0) 8	(14.7 ± 17.8) 8	P > 0.05
Diaphragm (n = 47)	(4.4 ± 7.4) 2	(6.7 ± 6.0) 4	
			P < 0.01

SD: standard deviation.

Table 4

Correlation of intensity of infection by *Sarcocystis* sp. between two different muscles (heart vs diaphragm) according to the technique employed, and between two different techniques (histology vs compression) according to the muscle analysed.

Survey (no animals)	Correlation y & x	r _s (95% C.I.)
Histology (n = 49)	Heart & diaphragm	0.249 (–0.043 to 0.502)*
Compression (n = 42)	Heart & diaphragm	0.317 (0.006–0.573)*
Heart (n = 51)	Histology & compression	0.582 (0.357–0.743)**
Diaphragm (n = 47)	Histology & compression	0.598 (0.367–0.759)**

n: Number of pairs.

r_s: Spearman rank correlation coefficient; C.I.: confidence interval.

*: P < 0.05; **: P < 0.01.

compression was significantly less sensitive than histology with diaphragm samples. Our results from the heart were comparable to the 77% found in northern Spain by compression (Hidalgo-Argüello

Table 5

Agreement in the detection (positive vs negative) of cysts of *Sarcocystis* sp. between heart and diaphragm according to the technique employed, and between histology and compression according to the muscle analysed.

Survey	Agreement y & x	Kappa (95% C.I.)
Histology (n = 49)	Heart & diaphragm	0.18 (–0.12 to 0.49)
Compression (n = 42)	Heart & diaphragm	0.00 (–0.24 to 0.24)
Heart (n = 51)	Histology & compression	0.42 (0.12–0.72)**
Diaphragm (n = 47)	Histology & compression	0.40 (0.17–0.63)**

n: Number of pairs; C.I.: confidence interval; **: P < 0.01.

et al., 2010), although they were much higher than the 30% reported in Hungary based on histology (Entzeroth et al., 1983). Considering the values obtained for diaphragm by compression our results showed a lower prevalence than that found in Poland (Tropilo et al., 2001: 94%), Lithuania (Malakauskas and Grikieniene,

Table 6

Age comparison of prevalence and median intensities detected by histology of heart in 59 animals classified as calves (N = 8), yearlings (N = 15) and adults (N = 36).

Age group	Prevalence n/N % (95% C.I.)	Median intensity
Calves	2/8 25 (3–65)	1
Yearlings	12/15 80 (54–94)	4
Adults	33/36 91 (78–97)	11
Statistical significance		
Age comparison	Upton's Chi square test	Wilcoxon test
Calves vs yearlings	$P < 0.05$	$P < 0.05$
Yearlings vs adults	—	—
Calves vs adults	$P < 0.01$	$P < 0.05$

C.I.: confidence interval.

—: No statistical significance.

2002: 84%) and Northern Spain (Hidalgo-Argüello et al., 2010: 73%) although higher than those obtained in a comparable area in Central Spain (Rojo-Vázquez et al., 2000: 8%). Higher intensities were observed in the heart irrespective of the method employed. These results agree with previous findings (Entzeroth et al., 1983; Malakauskas and Grikeniene, 2002; Hidalgo-Argüello et al., 2010) and support the role of the heart as a preferred location of *Sarcocystis* in red deer. However, recent investigations on *Sarcocystis* infection in this host in Norway report a noticeably higher tropism of *Sarcocystis* spp. for the diaphragm and oesophagus as compared to the heart (Dahlgren and Gjerde, 2010; Gjerde, 2014). Interestingly, the Norwegian authors did not find *S. cervicanis* morph type in their investigations of red deer, in contrast with findings of *S. cf. grueneri*, preferentially in heart samples, in other European countries (Entzeroth et al., 1983; Wesemeier and Sedlacek, 1995; Kutkienė, 2003) and of *S. cervicanis* in Spain (Hernández-Rodríguez et al., 1981; Hidalgo-Argüello et al., 2010). Other two morph types of *Sarcocystis*, referred to by Wesemeier and Sedlacek (1995) as *S. cf. capreolicanis* and *S. cf. hofmanni* and recently reclassified according to molecular characterization as *S. hjorti* (previously identified as *S. cf. capreolicanis*) (Dahlgren and Gjerde, 2010) and *S. elongata* and *S. truncata* (two species previously identified as *S. cf. hofmanni*) (Gjerde, 2014) have been frequently described in European red deer with preferential locations in diaphragm, oesophagus or both (Wesemeier and Sedlacek, 1995; Kutkienė, 2003; Dahlgren and Gjerde, 2010) and only occasionally in heart (Dahlgren and Gjerde, 2010; Gjerde, 2014). Two other species originally described in elk (*S. ovalis*) and reindeer (*S. hardangeri*) have been found in oesophagus and diaphragm from Norwegian red deer (Dahlgren and Gjerde, 2010). In the present study, of the two morph types identified in the sampled red deer, *S. cervicanis* was found in heart and diaphragm, but *S. hjorti* only in diaphragm. These results suggest that the heart is the preferred localization of *S. cervicanis*, whereas *S. hjorti* seems to have a preference for diaphragm. As a preliminary observation, *S. cervicanis* seems the predominant morph type in red deer population in this area although studies with more animals are needed.

Moderate indices of correlation and agreement were found between techniques. Accordingly, comparable diagnostic results (prevalence, intensity) with histology and compression were obtained for the heart analysis, although those in the diaphragm were contradictory since compression samples yielded higher median intensity but lower prevalence values. Freezing and thawing of the samples would presumably have affected both prevalence and intensity in a similar way. Thus, no explanation is presently

available for the discrepancy observed. Since comparison between fresh and frozen samples is of interest (e.g. retrospective studies), they should be investigated.

No agreement in the detection of the infection between the two muscles studied was found in our survey. Moreover intensities of the infection in both muscles showed a weak correlation. Using the compression technique, Malakauskas and Grikeniene (2002) observed a positive correlation ($P < 0.05$) between intensities of diaphragm, heart and oesophagus in other wild ungulates (roe deer, sika deer and wild boar), although they did not report the strength of that correlation and they could not investigate it in red deer because of the small number of heart and oesophagus samples. Unfortunately our paired results cannot be compared to other studies since as far as we know this is the first analysis of correlation and agreement between techniques and muscles in red deer.

Significantly lower infection prevalences and intensities were found in calves (<12 months age) whereas no differences were observed between yearlings and older animals. These results show that *Sarcocystis* infection in the sampled population of red deer is apparently age-related. The results are comparable to the higher indices described in adult deer (Goldová et al., 2008; Hidalgo-Argüello et al., 2010) and are suggestive of an incomplete protection against *Sarcocystis* re-infection. Moreover the age bias found should be considered in epidemiological surveys.

Taken together, our results suggest that the heart is the preferable tissue (relative to the diaphragm) for estimating prevalence and intensity of *Sarcocystis* spp. in red deer in the area studied. However, given that preliminary identifications revealed two morph types with different locations (*S. cervicanis* in heart and diaphragm, and *S. hjorti* only in diaphragm), a complementary sampling of diaphragm or oesophagus is advisable to avoid missing species like *S. hjorti*, with preference for other than heart muscles. Correlation and agreement between histology and compression were only moderate. While the sensitivity of compression and histology seems comparable in the analysis of heart, compression of diaphragm was significantly less sensitive and, in consequence, less useful. Therefore, both heart and diaphragm should be sampled and preferably assessed using histology to most reliably detect infection. The lower prevalence and intensity of *Sarcocystis* infection detected in calves should alert to a possible bias effect associated to the inclusion of these animals in epidemiological surveys.

Acknowledgements

This work has been funded by the Project FAU2006-00008-00-00. The authors are indebted to Dr. Bjorn Gjerde for his valuable advice. The authors also thank C. Rodríguez-Vigal and the staff of “Quintos de Mora” park.

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